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The localization, protein interaction, and cell surface mobility of neuronal α7 nicotinic acetylcholine receptors

ABHANDLUNG

zur Erlangung des Titels

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Table of Abbreviations

5-HT_3R	5-hydroxytryptamine$_3$ or serotonine receptor
α-BT	alpha bungarotoxin
α7 nAChR	alpha7 nicotinic acetylcholine receptor
aa	amino acid
AA	Alanine-Alanine
ACh	acetylcholine
AChE	acetylcholine esterase
AD	Alzheimer's disease
AF	Alexa Fluor® dye
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AP	action potential
AVI	audio video interleave
BSA	bovine serum albumin
CA1	*Cornu Ammonis* area 1
CCD	charge-couple device
cDNA	complementary deoxyribonucleic acid
ChAT	choline acetyltransferase
CNS	central nervous system
COS cells	kidney cell line from the African green monkey
D	diffusion coefficient
DA	dopamine; 4-(2-aminoethyl)benzene-1,2-diol
DIC	differential interference contrast
DIV	days *in vitro*
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
E18	embryonic day 18
EDTA	ethylene diamine tetraacetic acid
EGFP	enhanced green fluorescent protein
EGTA	ethylene glycol tetraacetic acid
EM	electron microscopy
ER	endoplasmatic reticulum
EYFP	enhanced yellow fluorescent protein
FCS	fetal calf serum
FRAP	fluorescence recovery after photobleaching
GABA	γ-aminobutyric acid
$GABA_AR$	γ-aminobutyric acid type A receptor
GAD	glutamate decarboxylase
GFP	green fluorescent protein
Glu	glutamate
GluR	glutamate receptor
Gly	glycine
GlyR	glycine receptor
GST	glutathione S-transferase
HA	anti-hemagglutinin
HEK cells	Human Embryonic Kidney 293 cells
His-tag	hexa histidine-tag
HRP	horse radish peroxidase
IgG	immunoglobulin G
IP	immunoprecipitation
IPSC	inhibitory postsynaptic current
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IR	immunoreactivity
IU	international unit
KO	knock out
LTP	long term potentiation
mAChR	muscarinic acetylcholine receptors
MEM	minimal essential medium
MLA	methyllycaconitine citrate hydrate
MSD	mean square displacement
nACHR	nicotinic acetylcholine receptor
NGS	normal goat serum
NMDAR	*N*-methyl *D*-aspartate receptor
NMJ	neuromuscular junction
PBS	phosphate buffered saline
PC	phase contrast
PDZ	**P**SD95 **D**lgA **z**o-1
PFA	paraformaldehyde
PKCα	protein kinase C alpha
PNS	peripheral nervous system
PSD	postsynaptic density
QD	quantum dot
QDOT	quantum dot
RNA	ribonucleic acid
RT	room temperature
SEM	standard error of the mean
SA	self-administartion
SFK	src-family kinases
shRNA	short hairpin ribonucleic acid
SPT	single particle tracking
SV	Sindbis virus
TM	transmembrane
TTX	tetrodotoxin
VDCC	voltage-dependent Ca^{2+} channels
VDSC	voltage-dependent Na^{+} channels
VGAT	vesicular γ-aminobutyric acid transporter
vGlut	vesicular glutamate transporter
VIAAT	vesicular inhibitory amino acid transporter
VTA	ventral tegmental area
WT	wild type
YTH	yeast two-hybrid

Summary

In the brain, alpha7 neuronal nicotinic acetylcholine receptors (α7 nAChRs) have a special role amongst nAChRs. α7 nAChRs are forming homopentamers, they display a high permeability for Ca^{2+}, and they are the most prevalent nAChRs in the brain. α7 nAChRs are found at the highest concentration in the hippocampus where they are located mostly on GABAergic interneurons and play an important role in learning and memory. Moreover α7 nAChRs have been involved in diseases such as Alzheimer's disease (AD) and schizophrenia, and are attracting considerable scientific interest to elucidate their contribution to disease mechanisms. While central cholinergic circuits have been investigated very extensively, the cell- and molecular biological properties of α7 nAChRs have not been studied in depth. The exact subcellular localization of α7 nAChRs is still debated, in particular in relation to synaptic sites, and only two proteins interacting with α7 nAChR, namely RIC-3 and Src-family kinases, have been identified to date. However, none of them is involved in synaptic clustering of α7 nAChR. In chapter 2 we describe the discovery of PICK1 as a novel α7 nAChR interacting protein. Thereby the PDZ domain of PICK1 binds to the large cytoplasmatic loop of α7 nAChR. We present evidence that PICK1 regulates clustering of α7 nAChRs in rat hippocampal interneurons.

The more detailed investigation of α7 nAChR clustering and surface dynamics demanded the ability to express exogenous gene constructs in cultured neurons. In chapter 3 we report an optimized transfection protocol for rat hippocampal neurons. Use of the magnetofection technique allowed the parallel transfection of several constructs and their expression in neurons for up to 3 weeks *in vitro*.

To understand the clustering and localization of α7 nAChRs it is necessary to investigate the surface dynamics of single receptors. In chapter 4 we report a detailed analysis of α7 nAChR cell surface mobility, using α-BT and QDots labeled single receptor trafficking. α7 nAChRs were found to be very mobile within the membrane. Clusters were found to be mobility traps, suggesting α7 nAChRs interact with underlying scaffolding proteins at these sites. Mobility

traps were found extrasynaptically and perisynaptically in close vicinity to GABAergic and glutamatergic postsynaptic densities. While extrasynaptic α7 nAChRs might activate Ca^{2+}-dependent signaling pathways, the perisynaptic α7 nAChRs are probably playing a modulatory role in GABAergic and glutamatergic synaptic activity. α7 nAChR mobility was not only dependent on localization but also on chronic synaptic activity changes and activation of the receptor itself.

Taken together, in this thesis work we identify α7 nAChR as a highly regulated receptor. The sites of α7 nAChR-dependent Ca^{2+} influx are tightly controlled by the cell. α7 nAChRs are clustered at distinct sites, reflecting functional heterogeneity. We identify for the first time a direct protein-protein interaction mechanism involved in the regulation of α7 nAChR clustering and possibly surface expression. We uncover α7 nAChR clusters as sites where mobility is constrained, but single receptors are able to diffuse in and out, confirming receptor clusters as steady-state receptor aggregations. We find α7 nAChR distributed all over the cell surface with clusters formed at extra- and perisynaptic sites. We speculate that α7 nAChRs have a variety of different functions dependent on their localization. The distinct mechanisms of the particular α7 nAChR subpopulations remain unclear, and are left to be addressed in future work.

Zusammenfassung

Alpha7 neuronale nikotinische Azetylcholinrezeptoren (α7 nACh Rezeptoren) unterscheiden sich im Gehirn von anderen nikotinischen Azetylcholin-rezeptoren durch mehrere Eigenschaften. Zum Einen sind α7 nACh Rezeptoren die häufigsten Azetylcholinrezeptoren im Gehirn. Zum Anderen sind die homopentameren Rezeptoren äusserst permeabel für Kalziumionen. Die höchste Dichte von α7 nACh Rezeptoren finden wir im Hippocampus auf GABAergen Interneuronen wo sie eine wichtige Rolle in Lern- und Gedächtnisprozessen spielen. Weiter wurde der α7 nACh Rezeptor als Akteur in verschiedenen Krankheiten, wie Alzheimer-Krankheit und Schizophrenie, identifiziert. Dies hat zu vermehrten Anstrengungen für die Aufklärung der Funktionsweise des Rezeptors geführt. Während zentrale cholinerge Vernetzungen ausführlich untersucht wurden, ist die Bedeutung von α7 nACh Rezeptoren auf Zell- und Molekularebene weitestgehend unklar. Die genaue subzelluläre Lokalisierung von α7 nACh Rezeptoren, speziell in Hinsicht auf die synaptische Umgebung, ist bis heute umstritten. Zudem wurden bis heute nur zwei Interaktionsproteine von α7 nACh Rezeptoren identifiziert. Jedoch scheint keines der beiden Interaktionsproteine, RIC-3 und Kinasen aus der Src-Familie, einen Einfluss auf die synaptische Aggregation von α7 nACh Rezeptoren zu haben. In Kapitel 2 beschreiben wir die Entdeckung von PICK1 als ein weiteres interagierendens Protein. Wir zeigen, dass die Interaktion über die PDZ Domäne von PICK1 und dem grossen zytoplasmatischen Teil des α7 nACh Rezeptors abläuft. Zudem zeigen wir, dass PICK1 die Aggregation des α7 nACh Rezeptors in hippocampalen Interneuronen aus der Ratte reguliert.

Eine ausführliche Untersuchung der Aggregation und der Oberflächendynamik des α7 nACh Rezeptors verlangt nach der Möglichkeit fremde Gensequenzen in kultivierten Neuronen zu exprimieren. In Kapitel 3 beschreiben wir ein solches optimiertes Transfektionsprotokoll für hippocampale Neuronen aus der Ratte. Die Verwendung der Magnetofektionstechnik erlaubte uns die parallele Transfektion und

Expression für bis zu 3 Wochen von mehreren Konstrukten in *in vitro* kultivierten Neuronen.

Um die Aggregation und die Lokalisation des α7 nACh Rezeptors zu verstehen, ist es notwendig die Oberflächendynamik eines einzelnen Rezeptors zu untersuchen. Im Kapitel 4 berichten wir in einer ausführlichen Analyse von der Zelloberflächenmobilität des α7 nACh Rezeptors. Dabei wurden einzelne Rezeptoren mit α-BT und QDots markiert. Wir identifizierten den α7 nACh Rezeptor als sehr mobilen Rezeptor innerhalb der Membran. Aggregationen von α7 nACh Rezeptoren kommen durch lokale Mobilitätsverringerung des Rezeptors zustande, was eine Interaktion zu zytosolischen Gerüstproteinen vermuten lässt. Solche Effekte konnten wir in extrasynaptischen wie auch perisynaptischen Stellen, nahe zu GABAergen und glutamatergen postsynaptischen Dichten, finden. Während die extrasynaptischen α7 nACh Rezeptoren wahrscheinlich kalziumabhängige Signalkaskaden aktivieren, haben die perisynaptischen α7 nACh Rezeptoren möglicherweise eine modulatorische Funktion. Zusätzlich zur Lokalisation hat auch die chronische synaptische Aktivität und die Aktivierung des Rezeptors selbst einen Effekt auf die Oberflächenmobilität.

Zusammengefasst, in dieser Arbeit identifizieren wir den α7 nACh Rezeptor als stark regulierten Rezeptor. Die Lokalisation des Rezeptors und somit der rezeptorabhängige Kalziumeinstrom wird durch die Zelle streng kontrolliert. Die unterschiedlichen Lokalisationen des Rezeptors lassen eine funktionale Heterogenität des α7 nACh Rezeptors vermuten. Wir beschreiben erstmalig eine Proteininteraktion mit dem α7 nACh Rezeptor welche die Rezeptoraggregation und dessen Oberflächenexpression reguliert. An Stellen wo die Rezeptormobilität stark eingeschränkt ist, beobachten wir nACh Rezeptoraggregationen. Einzelne Rezeptoren diffundieren jedoch in und aus der Aggregationstelle, so dass die Aggregation statt als fixes Gebilde besser als stetiger Zustand beschrieben werden sollte. Wir finden α7 nACh Rezeptoren diffus und aggregiert in extrasynaptischen und perisynaptischen Stellen. Wir vermuten, dass α7 nACh Rezeptoren je nach Lokalisation unterschiedliche Funktionen wahrnehmen. Die Aufklärung dieser Funktionen wird Teil zukünftiger Forschung sein.

1. Introduction

Neurotransmitter receptors are fundamental components of neuron-neuron communication. The localization and density of neurotransmitter receptors on neurons in the central and the peripheral central nervous system (CNS, PNS) are tightly regulated. Neurotransmitter receptors are aggregated in clusters at sites of their function. Any dysfunction in this regulation leads to neurological adverse consequences as occurring in several diseases. In this thesis we focused on the alpha7 neuronal nicotinic acetylcholine receptor (α7 nAChR). We investigated the clustering and localization of α7 nAChR in neuronal hippocampal cultures. We identified binding partners of α7 nAChR and their effect on α7 nAChR clustering. By using single particle tracking methods we further shed light on the cell surface dynamics of single α7 nAChR in respect to the localization of α7 nAChR cluster as well as synapses.

1.1. Discovery of the acetylcholine receptor

1.1.a. Discovery of chemical synaptic transmission

The α7 nAChR is a member of the family of the nicotinic acetylcholine receptors. nAChRs played an important role in the historical discovery of chemical neurotransmission. After decades of wild speculations and theories about the working mechanism of nerve and muscle in the Renaissance, the work of Galvani and Matteucci elucidated for the first time that the nerve-muscle signal transduction is based on electrical propagation (Matteucci, 1842). Based on this finding Claude Bernard could show in 1850 by using curare that the electrical impulses are transferred from the nerve to the muscle (Bernard, 1850). Just a few years later, in 1866, Vulpain suggested that the nerve and the muscle are not one unit. He proposed the existence of a neuromuscular junction (NMJ) between the nerve and the muscle (Vulpain, 1866). How the signal from the nerve overcomes this gap remained unclear until John Langley was investigating the effect of nicotine and curare 1905. Based on his findings he suggested that a transmitter-substance is released at the nerve terminal, propagating the signal to the muscle (Langley, 1905). Still the natural ligand was unclear. By 1914 Henry Dale isolated a compound

secreted by fungus ergot (*Claviceps*), which he identified as acetylcholine (ACh) (Dale, 1914b). He thoroughly investigated the actions of ACh and found that it produced two major responses: a ‘muscarine’ and a ‘nicotine’ action. Muscarine was isolated in 1869 by Schmiedeberg and Koppe from *Amanita muscaria*. They showed that muscarine arrests the beat of the frog heart (Schmiedeberg and Koppe, 1869). Dale described that the muscarine actions of ACh were antagonized by atropine, whereas the nicotine actions were antagonized by curare. Influenced by the work of his colleague Otto Loewi, he could show by extensive studies that ACh is a neurotransmitter on the smooth muscle cell but also at synapses at the autonomic ganglia. For the 'discovery of chemical synaptic transmission' he and Otto Loewi were awarded with the Nobel Prize in Physiology and Medicine in 1936.

1.1.b. Isolation and characterization of the nAChR

After the identification of ACh as a neurotransmitter tremendous work has been done to identify competitors of ACh. The presence of an acetylcholine receptor protein was speculated (Nachmansohn, 1955). But only with the discovery of α-bungarotoxin, a cholinergic antagonist isolated from the snake venom from *Bungarus multicinctus* (Chang and Lee, 1963), Changeux et. al finally managed in 1970 to confirm the existence of an ACh receptor in preparations of the electric organ of *Electrophorus electricus* (Changeux et al., 1970). According to the binding properties of nicotine, the receptor was termed nicotinic acetylcholine receptor. Further progress in the isolation of nAChRs of *Electrophorus electricus* (**Fig. 1-1**) (Olsen et al., 1972) allowed to propose that the nAChR is a pentameric protein (**Fig. 1-2**) (Hucho and Changeux, 1973). With the development of new techniques such as reverse genetics and molecular cloning the pentameric *Torpedo californica* nAChR was found to consist of related but genetically distinct subunits (**Fig. 1-1**). The subunits were classified according to similarities in protein sequences in α, β, δ, ε and γ subfamilies (Noda et al., 1982; Claudio et al., 1983; Noda et al., 1983a; Noda et al., 1983b; Takai et al., 1985). In contrast to non-α subunits the α subunit comprises a Cys-Cys pair at the extracellular side near to the transmembrane region 1 (TM1) which is crucial for ligand binding (Karlin et

al., 1986). Screening of brain cDNA libraries led to the discovery of several closely related sequences which encode 16 structurally homologues subunits. Further the subunits were subdivided into muscle or neuronal subunits. In muscle only two subunit compositions of nAChRs were discovered, that are 2α1, β1, γ, δ, and 2α1, β1, ε, δ. Whereas in neuronal tissue several α subunits (α2 - α7 and α9 - α10; α8 has been discovered in avian libraries but not in mammals) and β subunits (β2 - β4) were identified (Le Novere et al., 2002). In contrast to the muscle AChRs the neuronal AChR subunits form more versatile pentamers. α7-α10 exist each as homopentamers or as α7/α8 and α9/α10 heteropentamers. α2-α6 and β2-β4 are included in a range of complex heteromers (Le Novere et al., 2002).

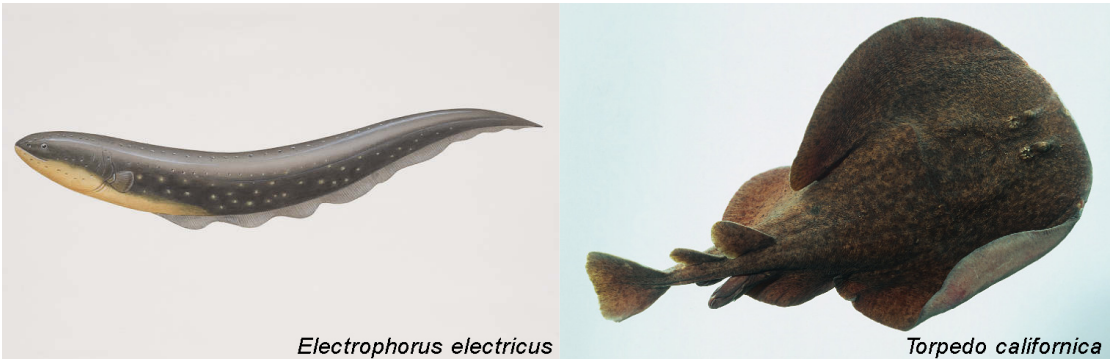

Fig. 1-1 Discovery of nAChR: The nAChR was first isolated from the electric organ of *Electrophorus electricus*. Another source of nAChR is the electric tissue of *Torpedo californica*.

One has to note that even though most of the neuronal nAChR subunits are expressed in neurons, they are also found in some non-neuronal cells throughout the body (Kawashima and Fujii, 2003; Gahring and Rogers, 2005). Further it is important to mention at this point that apart from nicotinic AChRs another group of ACh receptors exist, muscarinic acetylcholine receptors (mAChR) named after their binding affinity of muscarine. In contrast to nAChRs, which are ligand gated ion channels, the mAChRs are G protein coupled receptors which have slower and longer lasting effects. mAChRs activate downstream signaling via activation of phosphoinositide-specific phospholipase Cβ and Ca^{2+} release from internal Ca^{2+} stores (Eglen, 2006). Moreover, mAChRs are also involved in inhibition of elevated adenylate cyclase activity.

The studies with *Torpedo californica* nAChRs revealed the structural characteristics which are relevant to all nAChR subunits identified to date. Generally, all nAChRs assemble in a pentameric arrangement forming an internal membrane spanning pore which is permeable to a different degree for Na^+, K^+ and Ca^{2+}. Each subunit consists of 4 TM regions with the 3rd and 4th TM region being linked by a large cytoplasmatic loop. The N- and the C-terminals are extracellular (**Fig. 1-2**). Though the high structural similarities of the different subunits, nAChRs formed by the set of α, β, δ, γ, ε subunits feature great differences in ligand affinity (or anta-, agonist affinity), ion permeability, ion selectivity, and desensitization rate (Le Novere et al., 2002). Tissue specific nAChR subunit expression ensures adapted function of cholinergic receptors dependent on their localization.

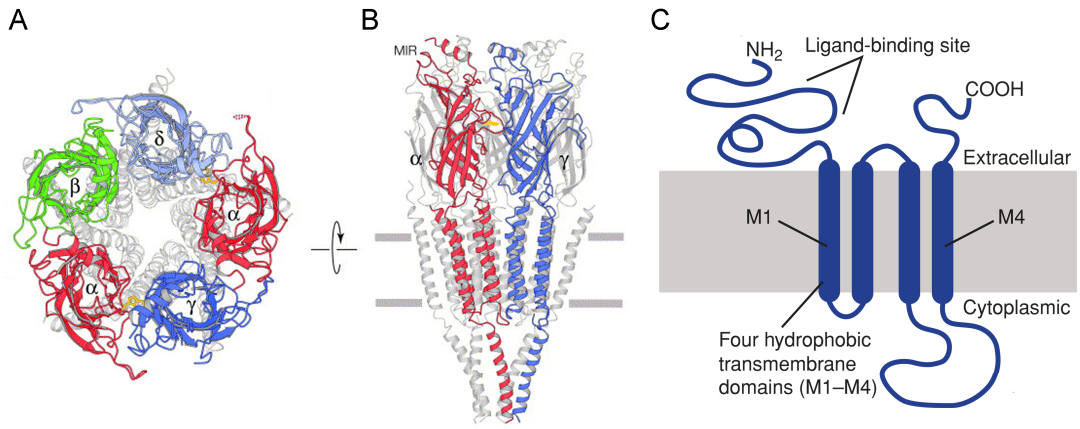

Fig. 1-2 Architecture of the nAChR: Ribbon diagrams of the whole *Torpedo californica* acetylcholine receptor at 4Å resolution. Viewed **(A)** from the synaptic cleft and **(B)** parallel with the membrane plane. In **(A)** the transmembrane pore formed by the 5 subunits is clearly visible. For clarity, only the ligand-binding domain is highlighted in **(A)** and only the front two subunits are highlighted in **(B)**. The grey bars in **(B)** indicate the membrane. Schematic drawing of a single subunit **(C)**. The subunit spans the membrane 4 times. The 3rd and 4th TM element are connected by a large cytoplasmatic loop. The C- and N-terminal are extracellular. The ligand binding site is located at the N-terminal of the α-subunits. (Unwin, 2005)

1.2. Principles of neurotransmission

1.2.a. From action potential to muscle contraction

The discovery of the neuromuscular junction (NMJ) as the first observed synapse, the relative large size of the synaptic apparatus and the well established purification methods were reasons for the NMJ to become a

model system in synapse research. In contrast to synapses in the central nervous system (CNS) the NMJ connects two very different types of cell types, namely neuronal cells and muscle cells. Nevertheless the NMJ is a perfect system to illustrate the principles of neurotransmission. As in all types of synapses the NMJ harbors the key players of a synaptic contact. Basically, a synapse propagates an electric signal, an action potential (AP), from the presynapse to the postsynapse of a connected cell. Therefore the electrical signal is transformed at the cell-cell junction into a chemical signal by the release of neurotransmitters from the active zone into the narrow synaptic cleft. The neurotransmitters are detected in the neighboring cell by neurotransmitter receptors in the postsynaptic density (PSD). The PSD is a protein network harboring all components of the postsynaptic apparatus. Activation of the neurotransmitter receptor results in the opening of the internal pore and the influx of ions. The inward directed current results in the generation of an AP and propagation of the electric signal. At the NMJ the arriving AP opens presynaptic voltage-dependent calcium channels and Ca^{2+} flows into the motor neuron's presynaptic cytosol. The presynaptic Ca^{2+} influx triggers the fusion of neurotransmitter containing vesicles with the membrane eliciting the release of ACh into the synaptic cleft. ACh diffuses to the motor endplate where it specifically binds to nAChRs. nAChRs, which are ligand-gated ion channels, open and allow Na^{+} and Ca^{2+} influx according to their gradient from the extracellular lumen into the cytosol of the muscle cell. The ion influx leads to a depolarization of the muscle cell. The depolarization further opens voltage gated sodium channels. The influx of Na^{+} ions generates an action potential which spreads within the muscle cell. Consequently the sarcoplasmic reticulum releases Ca^{2+}, eliciting the contraction of the muscle (**Fig. 1-3**).

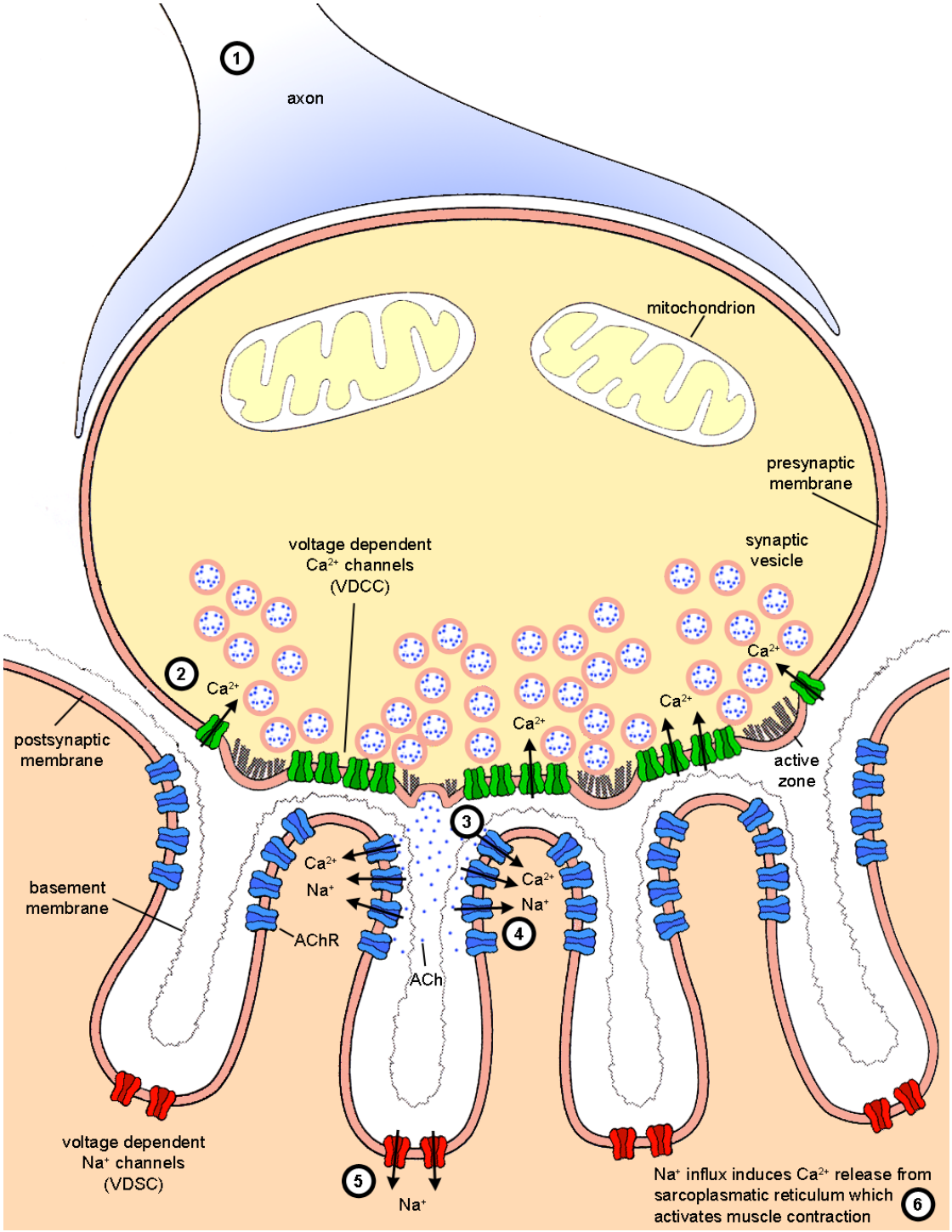

Fig. 1-3 Structure of the neuromuscular junction: Typical for the NMJ is the big terminal and the folded postsynaptic membrane. **(1)** An AP is propagated from the axon to the presynaptic terminal. **(2)** Voltage-dependent Ca^{2+} channels (VDCC) open. Ca^{2+} enters the presynaptic cytosol. **(3)** Primed synaptic vesicles, filled with ACh, fuse with the cell membrane. ACh is released into the synaptic cleft. **(4)** AChRs open upon ACh binding and allow Ca^{2+} and Na^{+} influx and membrane depolarization. **(5)** Voltage-dependent Na^{+} channels open. **(6)** Na^{+} influx induces Ca^{2+} release from the sarcoplasmatic reticulum which activates muscle contraction. Adapted in part from (Kandel et al., 2000)

1.2.b. NMJ vs. neuronal synapse

Even though the NMJ is a very useful system to investigate the architecture of a synapse and to uncover the molecular principles of neurotransmission, the system is limited. The NMJ differs in many aspects from a synaptic contact between neurons in the central nervous system (CNS). In comparison to the NMJ a neuronal synapse is around 1000 times smaller. The postsynaptic density (PSD) of the NMJ is folded several times to increase the surface allowing a higher receptor number in the synaptic cleft whereas the neuronal PSD has a small and unfolded surface and harbors just a fraction of the NMJ receptor number. In the folded synaptic cleft of the NMJ resides the basement membrane, or basal lamina (**Fig. 1-3**). A comparable structure in the neuronal synaptic cleft is absent (**Fig. 1-4**). The enzyme acetylcholine esterase (AChE), which degrades ACh to choline and acetate is aggregated in the basal lamina. While the NMJ PSD comprises only two types of nAChRs, the neuronal PSD can be excitatory or inhibitory, harboring glutamate receptors (GluR) and GABA receptors (GABAR), respectively. Accordingly, the neuronal synapses feature various types of presynapses, whereas the uniform NMJ presynapse stores only ACh-containing vesicles. In regard to the varieties between the two classes of synapses it is evident that the underlying PSD machinery is totally different. The differences between the NMJ and neuronal synapses reflect their functional specialization. The synaptic contact at the muscle end plate is optimized to propagate and amplify a single AP in the muscle with maximal reliability. In the CNS the focus lies less on the transmission of a single AP but rather on integration of multiple signals within the neuronal network and on the complexity of the network itself. In contrast to the simple architecture of motor neurons, the complex organization of neuronal networks demands regulatory mechanisms orchestrating excitatory actions.

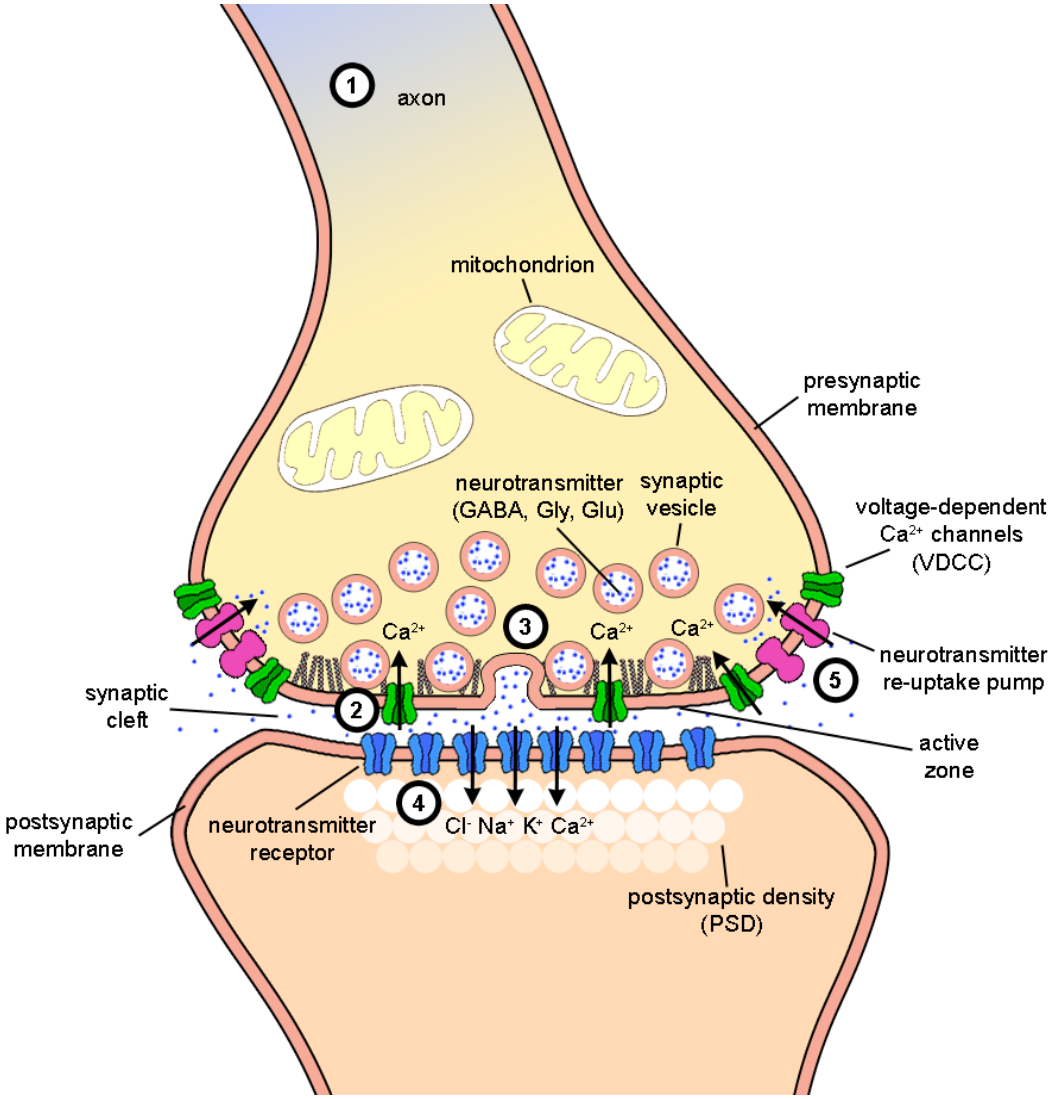

Fig. 1-4 Structure of a neuronal synapse: In contrast to the NMJ the postsynaptic membrane is not folded. Though, the mechanism of signal transduction is similar. **(1)** An AP arrives at the presynaptic terminal. **(2)** Voltage-dependent Ca^{2+} channels (VDCC) open and Ca^{2+} enters the cell. **(3)** Ca^{2+} triggers fusion of the neurotransmitter-filled synaptic vesicles with the plasma membrane. **(4)** Neurotransmitters enter the synaptic cleft. Upon binding of neurotransmitters to the neurotransmitter receptors, ions enter the postsynaptic cytosol. **(5)** Neurotransmitters are released from the receptor and diffuse away from the synaptic cleft where they get degraded or recycled by neurotransmitter re-uptake pumps.

1.2.c. An evolutionary point of view

During evolution from primitive animals with a simple neuronal network, such as jellyfish or worms, to species with a more complex and larger neuronal network and brain, the necessity for regulation of synaptic activity became more and more important. In early ancestor animals synaptic contacts were mainly excitatory whereas more complex species developed neuronal inhibitory mechanisms and synchronized neuronal networks to protect the brain from overexcitation. It has been shown that the proportion of cortical

inhibitory neurons correlates with the size of the brain (Glendenning, 1998). In the human brain inhibitory interneurons comprise 15-25% of the cortical neurons (Markram et al., 2004). Computational simulations and *in vivo* experiments brought evidence that the interplay of excitation and inhibition is extremely important to ensure the proper function of the brain (Dichter and Ayala, 1987; Vogels et al., 2005). A loss of inhibition leads to harmful epileptic activity.

In the course of evolution an ancestor ligand-gated cation permeable neurotransmitter, emerged through an early lateral transfer from a prokaryotic source 540 million years ago. By gene duplications and mutations the ancestor gave rise to a set of paralogs of structurally similar but functionally different neurotransmitter receptors (Tasneem et al., 2005). The paralogue receptors are oligomers forming a channel pore through the membrane allowing the selective influx of ions into the cytosol. Though the receptors exhibit different ion selectivity and are sensitive to different ligands their main structure is conserved supporting the hypothesis of a common origin. $GABA_AR$, nAChR, $5\text{-}HT_3$ and GlyR are all arranged as a pentamer with subunits harboring 4 TM regions. NMDAR, AMPAR and kainate receptor differ slightly from the pentameric receptors. Their subunits span the membrane 4 times but are arranged as a tetramer. In contrast to the structural similarities the functional differences are more pronounced among the set of ligand-gated ion channels. While NMDAR, AMPAR and kainate receptors are permeable for cations inducing excitation in a neuron, $GABA_AR$ and GlyR are inhibitory by allowing Cl^- ions to enter the cell and increasing the AP threshold level by hyperpolarization.

1.2.d. Architecture of a neuronal synapse

A theoretical neuronal network is built of a high number of neuronal units which are connected.

In the CNS a single neuron gets input from several thousands of other neurons. They release their neurotransmitters on synaptic receptors localized mainly on dendrites, causing excitation or inhibition. Excitation in the receiving neuron further results in the axonal release of cell type specific neurotransmitters onto hundreds of connected neurons. It is evident that a

complex neuronal network must be structurally organized and functionally tightly regulated. Therefore the arrangement and strength of synaptic contacts are crucial. In principal neurons of the neocortex the excitatory and inhibitory input on the dendrite is spatially separated (**Fig. 1-5**). Excitatory synaptic input from an axon is received on a dendritic membrane protrusion containing the postsynaptic machinery. These protrusions are called spines (**Fig. 1-6**). The dendrites of a single neuron possess from thousands up to a few hundred thousand spines. Spines incorporate the complete excitatory postsynaptic machinery.

The components of this machinery are centralized in the PSD. The glutamatergic PSD comprises dozens of different proteins with cytoskeletal and regulatory functions. PSD-95, Homer, Grip, and PICK1 are just a few of the many proteins playing a role in the structural matrix holding ion channels, kinases, and phosphatases in place, on the tip of the spine apposed to the active zone of the presynapse (**Fig. 1-5**). Spines maintain existing and establish new synaptic contacts with innervating axons.

Inhibitory postsynapses are fewer in number. The inhibitory PSD is in immediate proximity to the excitatory PSDs on the dendritic shaft of the same dendrite. There it receives axonal inputs from GABAergic or glycinergic axon terminals (**Fig. 1-5**). The close proximity of excitatory and inhibitory synapses requires a tight control of the localization of all synaptic proteins. The proper functionality of a synapse demands further that pre- and postsynapse are well aligned with a defined distance. Inconsistencies in the architecture of the synaptic cleft would affect the speed and probability of the AP induction. Additionally, the amount of released neurotransmitters and the number of opposing neurotransmitter receptors need to be balanced for synaptic transmission. To ensure that an AP and the following neurotransmitter release into the synaptic cleft overcomes the postsynaptic depolarization threshold level and provokes the generation of an AP in the postsynaptic cell, the number of responsive neurotransmitter receptors in the synaptic cleft has to reach a certain number. In addition to the receptor number the receptor localization has to be strictly regulated. Released neurotransmitters diffuse a few hundred nanometers in the synaptic cleft before they get enzymatically degraded or recycled. Therefore neurotransmitter receptors need to be

localized in immediate vicinity to the neurotransmitter release site. Receptor number and localization are two parameters which are tightly regulated by the postsynaptic cell. An imbalance in this regulation is known to cause diseases such as depression, schizophrenia, and addiction (Nader et al., 2006; Witkin et al., 2007; Kessler et al., 2009). The overall receptor number is adjusted by the rate of protein synthesis, rate of internalization and integration from and to the membrane, respectively. However, the receptor number in the PSD is dependent on specific anchoring mechanisms keeping the receptor at its place of function. The regulation of receptor number in the PSD goes in hand with the control of receptor localization. It has been shown that receptors are stabilized and anchored through different mechanisms (Allison et al., 1998). Receptors can be anchored directly or via an adaptor protein to cytoskeletal protein scaffolds. The actin cytoskeleton or the microtubule network provide the stability for receptor anchoring (**Fig. 1-5**). Another anchoring mechanism excludes cytoskeletal protein networks but consists of membrane patches with an interlaced protein network providing a scaffold for receptor stabilization.

Altogether, synaptic transmission at the synapse is dependent on many factors. Besides the complex presynaptic machinery guaranteeing tuned neurotransmitter release, the PSD is responsible for adjusted receptor number and localization which involves membranous or cytoskeletal scaffolding proteins interacting directly or indirectly with neurotransmitter receptors.

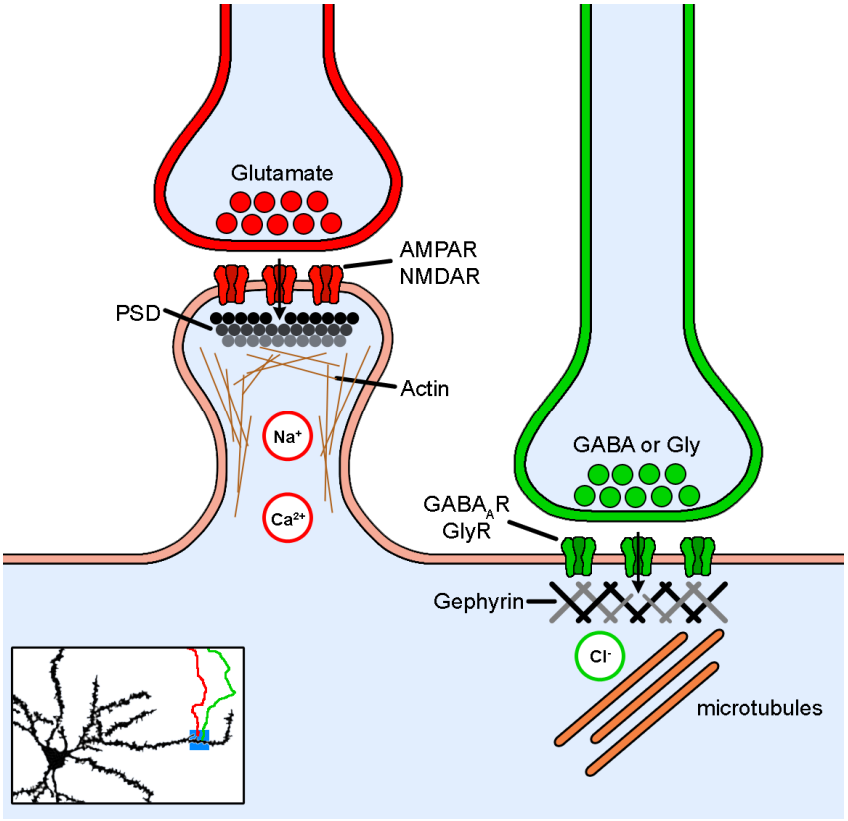

Fig. 1-5 Excitatory and inhibitory innervation: (left) Excitatory input is received from Glutamate (Glu) releasing terminals. Glutamate receptors (GluR) as AMPA receptors (AMPAR) and NMDA receptors (NMDAR) are located in spines. Actin ensures the shape of the spine while the postsynaptic density (PSD) protein network stabilizes the neurotransmitter receptors. Activation of AMPARs and NMDARs by Glu leads to Ca^{2+} and Na^{+} influx into the postsynaptic cytosol. **(right)** Inhibitory innervation takes place at the shaft of the dendrite. GABA or Glycin (Gly) is released into the synaptic cleft from the presynaptic terminal upon arrival of an AP. $GABA_A$ receptors ($GABA_AR$) and Glycinergic receptors (GlyR) are stabilized by Gephyrin and further unknown proteins. Following activation of $GABA_ARs$ or GlyRs, Cl^- enters and hyperpolarizes the cell.

1.3. From static to dynamic view

1.3.a. The tools for microscopy

In the past, all insights into the architecture of the NMJ and synaptic contacts in the brain were derived from microscopy studies. Thereby the tissue was stained with different dyes enhancing the contrast of tissue structures. Years later, antibody labeling further allowed elucidating the distribution of distinct proteins.

In the 19th and early 20th century stained tissue sections were mainly investigated with optical microscopes. The resolution of these analyses was limited due to the physical restriction of the wavelength of visible light. With the invention of the electron microscope in 1931 by Ernst Ruska, who was awarded with the Nobel Prize in Physics in 1986 for his discovery, biological samples could be investigated on an ultrastructural level. This fascinating tool

allowed the observation of single proteins and even lipid bilayers of the plasma membrane and vesicles (**Fig. 1-6**).

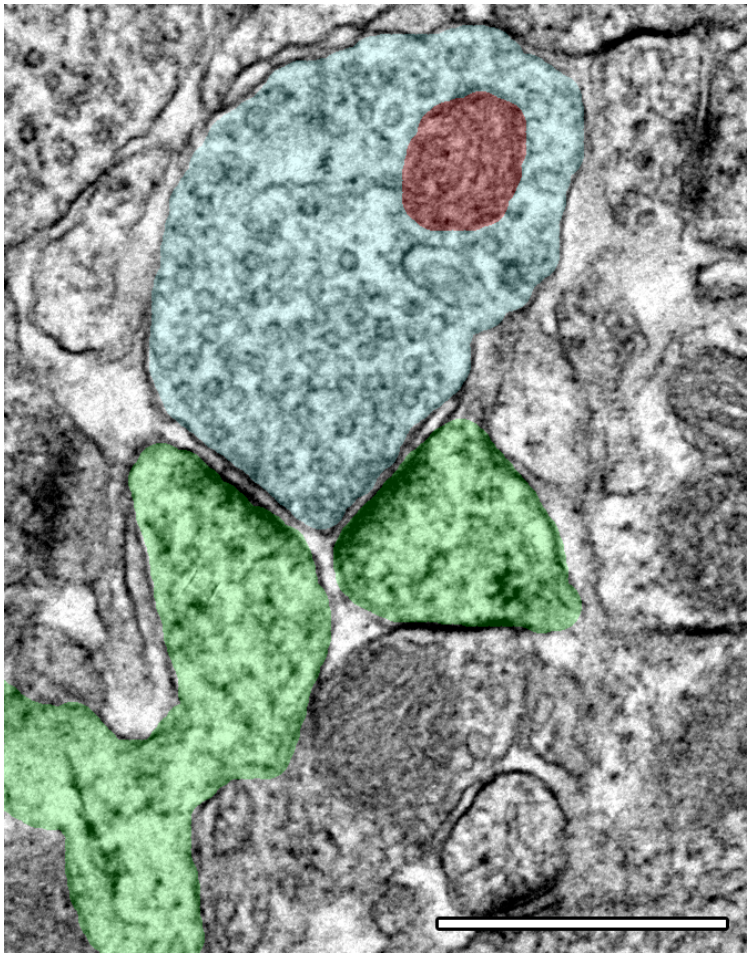

Fig. 1-6 Neuronal synapse viewed at with electron micrograph (EM): EM allows observations on an ultrastructural level. Darker areas are lipid rich structures whereas lighter areas are devoid of lipids. The image illustrates an excitatory synapse in the hippocampus. The large synaptic terminal is filled with synaptic vesicles **(blue)** and harbors one mitochondrion **(red)**. Two postsynaptic spines are opposed **(green)**. The PSD is clearly visible as dark band facing the synaptic cleft. Scale bar: 500 nm (Source: Caroline Petitjean)

Even though these methods reveal the structural architecture of cells, organelles, and also synapses, in an unprecedented detail-rich manner, the data just allow a static interpretation. This limitation motivated scientists to develop methods to collect in addition to spatial data also temporal data. The temporal visualization of a biological system provides important information about the dynamic processes taking place, providing a closer look at the real situation. The first live microscopy experiments were performed on large size specimens such as plant or animal cells without visualizing certain structures in the living cell. Deeper insights into cell processes such as protein localization, turnover and diffusion, or the dynamics of intracellular compartments such as endosomes became feasible with the discovery of the green fluorescent protein (GFP) and its use as a marker for protein expression (Chalfie et al., 1994). GFP is a fluorescent protein, which when exposed to blue light emits in the green spectra. It was isolated from jellyfish

(*Aequorea victoria*) in 1962 by Osamu Shimomura (Shimomura et al., 1962). In 2008 he and his colleagues Martin Chalfie and Roger Y. Tsien were awarded with the Nobel Prize in Chemistry for 'the discovery and development of the GFP'. GFP allows the detection of localization and movement of a protein of interest in living cells and even animals. Therefore, GFP is linked to the protein of interest by molecular cloning and expressed in cell culture or tissue. In the past years GFP was developed further and optimized, providing today a palette of different coloured fluorescent proteins and even a pH sensitive GFP. The use of GFP and its derivatives as markers in living cells established a new era of live microscopy research.

1.3.b. The brain – a dynamic structure

Live microscopy experiments with living neurons changed the understanding of synaptic contacts. With the acquisition of microscopy data in living neuronal tissue over a longer time period it became obvious that synaptic contacts are not rigid and inflexible structures, but rather that the neuronal network is very dynamic and underlies a constant change, even in the aged brain. Using two-photon microscopy and confocal microscopy dendritic spines were identified as very dynamic structures which change their shape within minutes and build up or brake synaptic contacts in the range of hours (Dunaevsky et al., 1999). These "plastic" changes in spine morphology and synaptic contact formation are mostly activity-dependent, displaying an adaptation mechanism reacting to neuronal activity or external input. Synaptic plasticity is believed to underlie motivation, learning, and memory (Hebb, 1949). On a closer look, synaptic plasticity is the strengthening or weakening of single synaptic contacts. This is regulated pre- or postsynaptically. Presynaptically, the modulation of the synaptic strength is achieved by regulation of the amount of neurotransmitter release per arriving action potential (Debanne et al., 2003). Postsynaptically, synaptic strength is controlled by the number and properties of neurotransmitter receptors (Debanne et al., 2003). The development of new tools to investigate single receptors within the membrane gave deeper insights into the membrane dynamics and the diffusion behaviour of receptors. Thereby single receptors are labelled with small fluorescent probes, composed of a semiconductor material. These probes, known as

quantum dots (QDOTs), have a remarkable photo stability and high quantum yield (Chan and Nie, 1998). These two features permit the detection and recording of single receptors over a long time period using a light microscope. From the recorded trajectories, parameters including diffusion coefficient, confinement, and mean square displacement can be determined (Bannai et al., 2006). Earlier studies labelled single receptors with silicon beads which are much larger in size compared to QDOTs (Meier et al., 2001). In contrast to silicon beads, QDOT labelling of receptors does not alter the diffusion behaviour within the membrane. Slight changes of receptor diffusion were only observed within the synaptic cleft. Even though QDOT coupled receptors are able to enter the synaptic cleft, the freedom of mobility is reduced due to the bigger size of the QDOT-antibody-receptor complex (Groc et al., 2007; Triller and Choquet, 2008).

Single particle tracking experiments of different labelled neurotransmitter receptors like GlyR, AMPAR, and NMDAR revealed a highly dynamic diffusion within the plasma membrane (Dahan et al., 2003; Groc et al., 2004). Neurotransmitter receptors are accumulated and clustered at the postsynaptic density. In addition single receptors diffuse freely within the plasma membrane. These findings suggest that receptor clusters are sites where receptors are slowed down by interacting with membrane proteins or cytosolic scaffolding proteins. In view of the fact that single receptors are just trapped temporary within a cluster points to a steady-state model in which receptors are constantly exchanged with the surrounding. Apart from lateral membrane diffusion, receptor number and localization is also dependent on dynamic intracellular processes. After a receptor is synthesised at the endoplasmatic reticulum (ER) and processed at the Golgi complex it is transported to endosomes from where it is further directed to the membrane. There, neurotransmitter receptors, diffuse freely within the membrane and eventually get confined in synaptic sites where they are activated by presynaptically released neurotransmitters. In parallel to receptor integration, receptors are constantly internalized from the plasma membrane and either recycled via endosomes or degraded within lysosomes.

With the development of new tools and microscopes it became evident in the last decade that neurotransmitter receptors are not statically integrated in the

membrane and synaptic contacts are not rigid constructs. Instead postsynaptic proteins such as scaffolding proteins and neurotransmitter receptors are dynamically regulated and are constantly in motion. These findings resulted in the view of the brain as a highly adaptive and a permanently altering neuronal network. Additionally, the understanding and the treatment of several diseases caused by an imbalance in the synaptic composition are now better understood from a dynamic-model point of view.

1.4. Nicotinic acetylcholine receptors in the brain

1.4.a. The cholinergic system

nAChRs are present on autonomic neurons, namely preganglionic neurons of the parasympathicus, on adrenal chromaffin cells in the PNS, on motorneurons in the NMJ and on many neurons in the CNS. The nAChRs in the CNS are classified as neuronal nAChRs. The diversity of nAChR localization implies the different roles in cholinergic transmission. Cholinergic systems are involved in arousal, sleep-wakefulness, locomotor behaviour, learning and memory (Woolf, 1991). The neuronal nAChRs became of interest in the recent past. Before that time, the focus was on the main excitatory and main inhibitory circuits in the brain. The glutamate receptors AMPAR and NMDAR are the most prominent receptors involved in excitation while GABA is the main inhibitory neurotransmitter in the mammalian brain. In contrast, cholinergic circuits comprise just a minority of all circuits (Woolf, 1991). The finding of the involvement of cholinergic processes in diseases such as schizophrenia in the last years resulted in increased effort in the uncovering of the cholinergic system. This was supported by the development of sensitive immunohistochemical methods, using choline acetyltransferase (ChAT) as a marker for cholinergic cells. In addition to motorneurons, there are three major cholinergic subsystems that innervate nearly every neural area (**Fig. 1-7**). One system sends widespread projections from the pedunculopontine tegmentum and the laterodorsal pontine tegmentum to the thalamus and midbrain dopaminergic areas and also to the caudal pons and medulla (Woolf, 1991). The second cholinergic system arises from various basal forebrain nuclei projecting through the cerebral cortex and the

hippocampal formation (Woolf, 1991). These two systems provide broad and diffuse projections to wide areas of the brain. The third subsystem consists of striatal cholinergic interneurons which project throughout the striatum and the olfactory tubercule (Zhou et al., 2002).

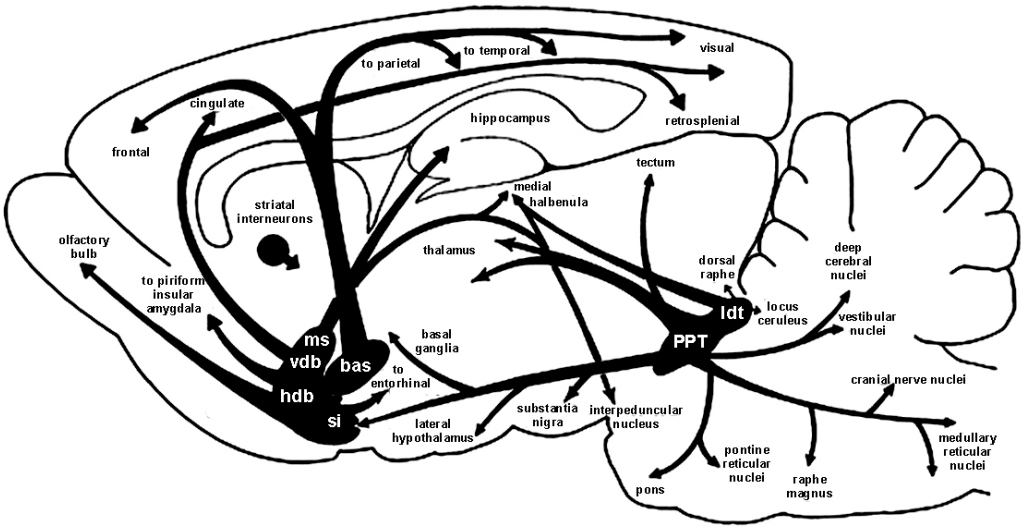

Fig. 1-7 Cholinergic circuits: The basal forebrain contains two groups of cholinergic neurons: (1) the medial septal group (medial septal nucleus **(ms)** and vertical diagonal band **(vdb)**) that project cholinergic axons to the hippocampus and parahippocampal gyrus. (2) The nucleus basalis group (nucleus basalis **(bas)**, substantia innominata **(si)** and horizontal diagonal band **(hdb)**) that project cholinergic axons to all parts of the neocortex, parts of limbic cortex and to the amygdala. The cholinergic pontomesencephalon neurons (laterodorsal tegmental **(ldt)** and pedunculopontine tegmental nuclei **(ppt)**) project onto hindbrain, thalamus, hypothalamus and basal forebrain. (Woolf, 1991)

In contrast to the main excitatory and inhibitory systems the cholinergic projections differ in their mode of transmitter release. While glutamate and GABA are released at synaptic sites, the majority of cortical and hippocampal cholinergic release sites are nonsynaptic (Umbriaco et al., 1994; Descarries et al., 1997). Nonsynaptic release and “spill over” of neutransmitters from synapses contribute to volume transmission which enables ACh to diffuse and act at lower concentrations some distance away from the release site. The concentration of extrasynaptic ACh is regulated by AChE, which degrades ACh to choline and acetate. The presence of AChE ensures therefore that volume transmission is not uncontrolled, but rather that extrasynaptic ACh is cleared from distinct areas (Kawaja et al., 1990).

1.4.b. The α7 nAChR – unique among the nAChRs

Among the many possible compositions of nAChRs in the brain, the α7 nAChR belongs to the most abundant variants, being just topped by the α4β2 nAChR. *In situ* hybridization studies detected high levels of α7 nAChR transcripts in olfactory areas, hippocampus, amygdala, and cerebral cortex (Seguela et al., 1993). Nevertheless most of the initial studies on α7 nAChR were done in the chick ciliary ganglion. The chick ciliary ganglion was favoured due to the size of the synapses, the high content of α7 nAChRs, and the easy access to the tissue. There, α7 nAChR is perisynaptically localized, excluded from the PSD (Jacob and Berg, 1983; Loring et al., 1985; Horch and Sargent, 1995).

Later studies focused on the hippocampal formation, where a fine network of cholinergic fibres innervates pyramidal cells, granule cells, interneurons, and neurons of the hilus (Frotscher and Leranth, 1985). But still, in addition to those direct synaptic contacts there is a high portion of volume transmission in the hippocampus (Umbriaco et al., 1994; Descarries et al., 1997). α7 nAChRs were best investigated in the hippocampus where they are most prominent on GABAergic interneurons. In addition, the α7 nAChR was found nearly on every synapse in the CA1 stratum radiatum of the rat hippocampus (Fabian-Fine et al., 2001). The largest size α7 nAChR-containing interneurons are found in CA3, in both stratum radiatum and stratum lucidum (Freedman et al., 1993). α7 nAChR differs in many aspects from other nAChRs. While the α4β2 nAChR has a high affinity for ACh but slow desensitization, the α7 nAChR binds ACh with low affinity but has an exceptionally fast desensitization (Quick and Lester, 2002). These properties make α7 nAChR a candidate responsive to volume transmission with low ACh levels featuring rare desensitization (Dani and Bertrand, 2007). Moreover α7 nAChR is unique in terms of structure and permeability. To date, α7 nAChR has been found to be the only homopentameric AChR in the brain with the highest affinity of all neuronal AChRs to α-BT (Couturier et al., 1990; Seguela et al., 1993). Further, the α7 nAChR differs strikingly from its homologues by displaying a much higher permeability of Ca^{2+} ions reaching values of the NMDAR in human brain

(Bertrand et al., 1993; Seguela et al., 1993). But unlike the NMDA-receptor, the α7 nAChR is permeable for calcium ions even at resting membrane potential. These features of α7 nAChR are highly conserved throughout the animal kingdom, pointing to a critical role of the receptor in neuronal networks (Le Novere et al., 2002).

1.4.c. Nicotine, a modulator of cognitive function

Tobacco smoking and acute and chronic administration of nicotine improve cognitive functions (Poincheval-Fuhrman and Sara, 1993). It has been shown that the improvement results from facilitated induction of long-term potentiation (LTP) in the hippocampus (Fujii et al., 1999), a synaptic model of learning and memory. The underlying mechanism is thought to be an ACh-dependent alteration of the oscillatory rhythms that result from the interaction of the various hippocampal subregions (Hasselmo, 2006). Altering such rhythms may result in an increase in the recruitment of various cell-signalling molecules known to be involved in synaptic plasticity as measured by LTP. Thereby different nAChR subtypes, mainly α7 and α4β2 nAChRs, modulate different types of LTP in the various subregions of the hippocampus (Kenney and Gould, 2008).

While in rats injection of the α7 nAChR antagonist MLA into the ventral hippocampus and basolateral amygdala impairs working memory (Levin, 2002), the administration of the partial α7 nAChR agonist GTS-21 significantly improves learning and memory in rats (Arendash et al., 1995) and also attenuates the age-related impairment of classical conditioning in rabbits (Woodruff-Pak et al., 2000). A possible mechanism of nicotine induced LTP acts through the desensitization of α7 nAChRs on inhibitory interneurons in the hippocampal CA1 by nicotine. The desensitization of α7 nAChR reduces GABAergic inhibition and therefore indirectly increases the excitability of pyramidal cells, leading to LTP induction (Fujii et al., 2000).

Additional proof for the importance of α7 nAChR in cognition was given with studies in α7 nAChR deficient mice. In comparison to wild-type (WT) mice, α7 nAChR knock-out (KO) mice showed significant deficits in attention and working memory. These behavioural deficits were associated with the loss of

α7 nAChR, as α4β2 nAChR density was unaltered in these mice (Young et al., 2007).

In addition to cognitive functions nicotine modulates also the dopaminergic (DA) system, which is involved in the reward circuits of the nucleus accumbens, an area involved in the development of addiction (Mansvelder and McGehee, 2002). α7 nAChR KO mice were used to investigate the role of α7 nAChR in nicotine addiction. Pons et al. showed that α7 nAChR KO mice do self-administer nicotine in a manner indistinguishable from WT mice (Pons et al., 2008). This is consistent with the finding that α7 nAChR is not involved in chronic nicotine self-administration (SA) in rats (Grottick et al., 2000). α7 nAChR is believed to play a key role in long-term adaptations to passive chronic nicotine SA (Besson et al., 2007; Salas et al., 2007). Recent work performed in slices showed that following short applications of nicotine, α4β2 nAChRs on ventral tegmental area (VTA) DA neurons and VTA GABAergic neurons desensitize. However α7 nAChRs on glutamatergic axons in the VTA do not desensitize and therefore potentiate glutamatergic excitation on DA neurons (Mansvelder and McGehee, 2000, 2002). As a consequence, DA neurons fire more frequently. The enhanced glutamatergic release produces LTP of the glutamatergic afferents contributing to the process of addiction (Dani and Harris, 2005). In addition to the existing evidence of the *in vitro* studies it is of note that a recent work showed that α7 nAChR KO mice exhibit only a slightly altered electrophysiological response to nicotine in DAergic neurons *in vivo*.

Altogether α7 nAChR involvement has been confirmed for cognitive functions such as attention and learning and memory. Although the implication of α7 nAChR in nicotine addiction is controversial, several other diseases are linked to α7 nAChR underlining its significance.

1.4.d. Mental diseases and the involvement of α7 nAChR

The improvement of cognitive functions due to nicotine administration was also perceived by patients suffering from schizophrenia. The prevalence of tobacco smokers in this group is significantly increased compared to healthy

subjects, pointing to a positive effect of nicotine attenuating the symptoms (de Leon et al., 1995). In addition to hallucinations and delusions, schizophrenia patients suffer from the ability to focus attention (Nuechterlein and Dawson, 1984). The latter symptom is regulated by α7 nAChR. Pharmacological and genetical studies identified the α7 nAChR as an important target in the treatment of schizophrenia (Freedman et al., 1994; Leonard et al., 2002). Patients with schizophrenia have altered expression and regulation of α7 nAChRs in the reticular nucleus of the thalamus, the hippocampus, the cingulated cortex and the frontal lobe regions (Freedman et al., 1995; Court et al., 1999; Guan et al., 1999; Marutle et al., 2001). The underlying mechanism in attention deficiencies in schizophrenic patients involves α7 nAChRs on GABAergic interneurons (Albuquerque et al., 1998; Frazier et al., 1998b). α7 nAChR induces GABA release which acts on $GABA_B$Rs thus decreasing the release of glutamate. This prevents hippocampal neurons acting properly in attention related tasks (Hershman et al., 1995). In addition to schizophrenia, the α7 nAChR has been implicated in a wide range of neuronal dysfunction and mental illness (Picciotto and Zoli, 2002; Gotti and Clementi, 2004).

Alzheimer's disease (AD) is the most common form of degenerative dementia affecting mainly elderly persons (Palmer, 2002). In AD patients a progressive loss of cognitive functions particularly in learning and memory is correlated with the neuronal loss in the cholinergic system (Coyle et al., 1983; Perry, 1986). The most dramatic symptoms of AD appear with the loss of cholinergic neurons in the cortex and the hippocampus (Nordberg, 1999; Perry et al., 2000). To date no cure is known for AD, but treatment of AD patients with AChE inhibitors counteract against the loss of cholinergic signalling by decreasing the hydrolysis rate of ACh (Maelicke et al., 2001). The treatment with nicotinic agents improves cognitive deficits of AD patients (Nordberg, 1999; Picciotto and Zoli, 2002). Amongst all AChRs the α7 nAChR became the main candidate playing a potential role in AD. α7 nAChR has been found to bind with high affinity to the $A\beta_{1\text{-}42}$ (Wang et al., 2000; Oddo and LaFerla, 2006). Further support for this prospect was given by the discovery of α7 nAChRs localized in amyloid plaques (Wang et al., 2000). There is accumulating evidence that $A\beta_{1\text{-}42}$ inhibits currents mediated by α7 nAChRs

(Liu et al., 2001a; Pettit et al., 2001). The inhibition by Aβ would explain the decreasing synaptic induction of LTP contributing to a cognitive decline (Freir et al., 2001). Additionally, transgenic AD mice displayed a loss of α7 nAChRs (Oddo and LaFerla, 2006). All these findings bear evidence that α7 nAChRs are directly involved in the cognitive decline during AD.

1.4.e. Where is α7 nAChR?

To further identify and understand the mechanisms of α7 nAChR involvement in neuronal diseases and LTP induction, the knowledge of the receptor's cellular localization and the molecular interaction partners is crucial.

Up to date, α7 nAChRs have been identified in postsynaptic, presynaptic, perisynaptic, and extrasynaptic sites. In the hippocampus and neocortex, notably on GABAergic interneurons (Freedman et al., 1993), α7 nAChRs mediate cholinergic postsynaptic input (Frazier et al., 1998a) and enhance GABAergic IPSCs in principal neurons (Frazier et al., 2003). Ultrastructural studies reported their presence in glutamatergic postsynaptic sites on neurons of the somatosensory cortex (Levy and Aoki, 2002; Jones et al., 2004) but also in a perisynaptic annulus around dendritic glutamatergic postsynaptic spines on cortical pyramidal cells (Fabian-Fine et al., 2001). Possible functions of postsynaptic α7 nAChR are assistance of NMDAR activation and LTP induction by removal of the Mg^{2+} block, a modulatory role by Ca^{2+} influx activating 2nd messenger pathways, and less likely depolarization of the membrane by α7 nAChR itself. Besides postsynaptic α7 nAChR, the receptor is also located presynaptically. There it facilitates vesicle fusion and the release of various neurotransmitters (McGehee et al., 1995; Alkondon et al., 1997; Li et al., 1998; Radcliffe and Dani, 1998; Maggi et al., 2001; Jones et al., 2004). In primary hippocampal cultures, α7 nAChRs are prominent in interneurons, forming somato-dendritic clusters, partially localized at GABAergic synapses (Kawai et al., 2002). Recently, several lines of evidence indicated that α7 nAChRs are expressed extrasynaptically in the CNS (Fabian-Fine et al., 2001; Jones and Wonnacott, 2004). Extrasynaptic α7 nAChR may be targeted by ACh released into the extrasynaptic space. It has been shown that activation of extrasynaptic α7 nAChRs by cholinergic

agonists either facilitates or depresses backpropagating action potentials, depending on the timing of α7 nAChR activation (Rozsa et al., 2008). To localize α7 nAChR at various sites, mechanisms are needed to target the receptor specifically. Adaptor proteins interacting with receptors are known to fulfil such a task. E.g. Gephyrin is the scaffolding protein of $GABA_A$Rs stabilizing the receptor in inhibitory postsynapses (Essrich et al., 1998).

No α7 nAChR interacting proteins apart from RIC-3 and Src-family kinases (SFKs) have been identified so far. However RIC-3 is thought to be responsible for the correct folding of α7 nAChR. Therefore RIC-3 is probably not involved in membrane localization of α7 nAChR (Millar, 2008). SFKs have been shown to phosphorylate α7 nAChRs, causing decreased receptor activity (Charpantier et al., 2005). So far interaction partners regulating localization and clustering of α7 nAChR are unknown.

Taken together, α7 nAChR has been found in synaptic and non-synaptic sites. The evidence for presynaptic α7 nAChR is based on many studies using different approaches, whereas the postsynaptic localization of α7 nAChRs is still discussed. α7 nAChRs identified in postsynapses by immunogold labeling are located certainly in noncholinergic synapses, presumably GABAergic or glutamatergic, because they include nearly all of the recognizable synapses in the CA1 region (Fabian-Fine et al., 2001). Furthermore, α7 nAChRs cluster at GABAergic synapses on cultured hippocampal neurons in postsynaptic location (Kawai et al., 2002). Despite all the published results showing α7 nAChR postsynaptically localized, the receptor may be viewed as perisynaptic where it modulates effective noncholinergic synapses (Berg and Conroy, 2002).

1.5. Aim

In this thesis we try to uncover the mechanisms regulating the membrane dynamics and the clustering of α7 nAChRs.

The clustering of a membrane protein is dependent on the stabilization by a direct or indirect link to scaffolding elements. The indirect link is performed by adaptor proteins binding to the membrane protein and scaffolding protein, thus forming a stable complex.

In chapter 2 we hypothesize the existence and function of a potential α7 nAChR interacting protein. Does this protein regulate α7 nAChR clustering and distribution? Which domains are essential for protein-protein binding? To address these questions we first identified α7 nAChR interacting proteins by a yeast two-hybrid assay, using the large cytoplasmatic loop of α7 nAChR as bait. PICK1, a protein known to interact with PKCα, was identified as α7 nAChR interacting protein. Immunoprecipitation (IP) experiments were performed in the rat brain and cell lines to investigate the protein-protein interaction and the involved protein domains. To focus on the functional role of PICK1 on α7 nAChR, PICK1 overexpression in rat hippocampal neurons using virus and lipofection techniques was carried out.

The regulation of receptor clustering and localization is consequently also influenced by the membrane diffusion of a receptor. Membrane proteins have been shown to diffuse within the membrane of the cell surface. Areas where diffusion is decreased are spots where membrane proteins aggregate. To understand the regulation of α7 nAChR clustering, it is not only important to know the interacting proteins but also to investigate the dynamics of the receptor within the membrane. To enable such an investigation, it is necessary to express different exogenous-tagged proteins in specific neurons. Transfected gene constructs can code for fluorescently-tagged proteins or for mutant proteins, used as markers or tools to interfere with cellular mechanisms, respectively. At the time I started my thesis work, there was no satisfying method known to transiently transfect cultured neurons with high efficiency and low toxicity. In chapter 3 we describe the development and optimization of a protocol for the efficient transfection of cultured neurons.

Having achieved the capability to transiently transfect neurons the investigation of surface α7 nAChR dynamics became possible. In chapter 4 we focused on α7 nAChRs in living neurons. We tackled the questions how mobile α7 nAChRs are in the cell membrane? Is the mobility affected by scaffolding elements such as microtubules or the actin network? At which sites is α7 nAChR mobility decreased? Is the mobility of α7 nAChR affected by neuronal activity? To answer these questions single α7 nAChRs were labeled with QDOTs, which are small fluorescent probes with a high quantum yield and high photostability, and tracked by live microscopy. Inhibitory and excitatory PSDs were visualized by the expression of fluorescently tagged marker proteins in the α7 nAChR positive neurons. Receptor mobility in correlation to their localization was analyzed by custom software.

In this thesis work we try to reveal the mechanisms of α7 nAChR clustering, possible interacting proteins, the underlying dynamic diffusion behavior of single α7 nAChR receptors, and their adaption to neuronal activity. The answers to these questions will hopefully help to clarify the ongoing debate on α7 nAChR and allow new speculations and hypotheses.

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List of Publications

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Postsynaptic mechanisms influence the formation of GABAergic synapses in hippocampal cultured neurons, Neuroscience 2009, submitted, *C. Petitjean, **T. Bürli**, C. Sidler, J.-M. Fritschy*

Efficient transfection of DNA or shRNA vectors into neurons using magnetofection. Nat Protoc. 2007;2(12):3090-101. ***T. Buerli** & C. Pellegrino et al.*

PICK1 interacts with alpha 7 neuronal nicotinic acetylcholine receptors and controls their clustering. Mol Cell Neurosci. 2007 Jun;35(2):339-55. Epub 2007 Mar 24. *K. Baer & **T. Bürli** et al.*

Caveolin-Stabilized Membrane Domains as Multifunctional Transport and Sorting Devices in Endocytic Membrane Traffic. Cell 2004, Volume 118, Issue 6, Pages 767-780 *L. Pelkmans, **T. Bürli**, M. Zerial, A. Helenius*

Analysis of interaction between caveolae-mediated endocytosis and endocytosis via clathrin coated pits. Diploma work 2003, ***T.Bürli***, supervised by Dr. A. Tagawa , Lab of Prof. Dr. A. Helenius, ETH Zürich

The effect of active and dominant negative mutants of RabGTPases on the entry of Simian Virus 40. Semester work 2002, ***T.Bürli***, supervised by Prof. Dr. L. Pelkmans, Lab of Prof. Dr. A. Helenius, ETH Zürich

Presentations

Federation of European Neuroscience Societies FENS 2008 (Genf)	Dynamics of alpha 7 neuronal nicotinic acetylcholine receptors.
Society for Neuroscience SfN 2007 (San Diego, USA)	Dynamics of alpha 7 neuronal nicotinic acetylcholine receptors.
Neuroscience Center Zürich 2007 (Zürich):	Dynamics of alpha 7 neuronal nicotinic acetylcholine receptors.
Swiss Society for Neuroscience SSN 2007 (Bern):	PICK1 interacts with alpha 7 neuronal nicotinic acetylcholine receptors and controls their clustering.
Swiss Society for Neuroscience SSN 2006 (Basel):	PICK1 interacts with alpha 7 neuronal nicotinic acetylcholine receptors and controls their clustering.
Neuroscience Center Zürich 2006 (Zürich):	PICK1 interacts with alpha 7 neuronal nicotinic acetylcholine receptors and controls their clustering.
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